Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8343–8347, August 1995
Biochemistry

The G-protein-coupled receptor phosphatase: A protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity

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Contributed by Robert J. Lefkowitz, May 17, 1995

ABSTRACT **Phosphorylation of G-protein-coupled receptors plays an important role in regulating their function. In this study the G-protein-coupled receptor phosphatase (GRP) capable of dephosphorylating G-protein-coupled receptor kinase–phosphorylated receptors is described. The GRP activity of bovine brain is a latent oligomeric form of protein phosphatase type 2A (PP-2A) exclusively associated with the particulate fraction. GRP activity is observed only when assayed in the presence of protamine or when phosphatase-containing fractions are subjected to freeze/thaw treatment under reducing conditions. Consistent with its identification as a member of the PP-2A family, the GRP is potently inhibited by okadaic acid but not by I-2, the specific inhibitor of protein phosphatase type 1. Solubilization of the membrane-associated GRP followed by gel filtration in the absence of detergent yields a 150-kDa peak of latent receptor phosphatase activity. Western blot analysis of this phosphatase reveals a likely subunit composition of $AB_{\alpha}C$. PP-2A of this subunit composition has previously been characterized as a soluble enzyme, yet negligible soluble GRP activity was observed. The subcellular distribution and substrate specificity of the GRP suggests significant differences between it and previously characterized forms of PP-2A.**

Exposure of the β_2-adrenergic receptor (βAR) receptor to agonists results in a rapid decline in receptor responsiveness, a process that appears to involve receptor phosphorylation (1, 2). In addition to the second messenger-dependent protein kinases (1), agonist-specific phosphorylation of this receptor can also be effected by the βAR kinase (βARK), a member of the family of second messenger-independent G-protein-coupled receptor kinases (GRKs).

Despite the considerable progress that has been made in identifying and characterizing the mechanism of action of βARK, little is known of the phosphatases responsible for reversing this phosphorylation event. Resensitization, presumably due to dephosphorylation of the βAR, has been shown to occur rapidly upon removal of agonists (3). Regulation of receptor phosphatase activity, therefore, represents an important potential mechanism for modulating receptor function.

In this study, we characterize the βARK-phosphorylated βAR phosphatase present in extracts of bovine brain. This phosphatase activity, which is also capable of dephosphorylating βARK-phosphorylated α_2C_2-adrenergic receptors (α_2C_2ARs) and rhodopsin kinase (RK)-phosphorylated rhodopsin, is termed the G-protein-coupled receptor phosphatase (GRP). The enzyme, an oligomeric form of protein phosphatase type 2A (PP-2A), is latent and specifically associated with the particulate fraction.

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MATERIALS AND METHODS

Preparation of ^{32}P-Labeled Substrates. Purified reconstituted βAR (200 nM) (4–6) was phosphorylated (500-μl reaction volume) with purified βARK, βAR kinase 2 (βARK-2), RK (7), or cAMP-dependent protein kinase (PKA) (125 nM) as described (8). Reactions were terminated after incubation at 30°C for 20 min by addition of 1 ml of ice-cold 50 mM Tris·HCl, pH 7.0/0.1 mM EDTA/50 mM 2-mercaptoethanol (buffer A). Labeled receptor was centrifuged (350,000 × *g* for 15 min), washed three times, and resuspended in buffer A. Stoichiometries of phosphorylation were 2–4 mol of P_i per mol of βAR for βARK or βARK-2, 1–2 mol of P_i per mol of βAR for RK, and 1.5–1.7 mol of P_i per mol of βAR for PKA. Purified Sf9 cell membranes containing human βAR or α_2C_2AR and rod outer segment membranes (9, 10) were phosphorylated as described for the purified βAR. The βAR and α_2C_2AR were phosphorylated by using the βARK and rhodopsin was phosphorylated by using RK to stoichiometries of 3.0, 3.2, and 4.0 mol of P_i per mol of receptor, respectively. Phosphorylase *a* was prepared as in ref. 11.

Phosphatase Assays. To assess GRP activity, ^{32}P-labeled βAR (1.0 pmol) was incubated with a source of phosphatase in buffer A in 30 μl. Incubations were performed at 30°C for the times indicated. Dephosphorylation reactions were terminated by the addition of SDS sample loading buffer and proteins were separated on SDS/10% polyacrylamide gels. Phosphorylated βAR was visualized by autoradiography and quantified by phospho imager analysis. Okadaic acid (OA) (5 μM) was included in a control incubation to eliminate the possibility that proteolysis contributes to the loss of ^{32}P-labeled receptor.

Phosphorylase *a* phosphatase assays were performed as described in ref. 12. To determine protein phosphatase type 1 (PP-1) and PP-2A activities, 1 nM OA was used to inhibit PP-2A and 100 nM I-2, the recombinant rabbit skeletal muscle inhibitor 2 (13), was used to inhibit PP-1. I-2 at 100 nM and OA at 1 nM or OA at 5 μM were used to inhibit both PP-1 and PP-2A. Where indicated phosphatase-containing fractions were subjected to freeze/thaw treatment in the presence of 0.2 M 2-mercaptoethanol prior to assay.

Sources of Protein Phosphatases. Frozen bovine cerebral cortex (100 g) was homogenized in 500 ml of 50 mM Tris·HCl, pH 7.0/5 mM EDTA/50 mM 2-mercaptoethanol (buffer B) at 4°C and passed through a double layer of cheesecloth. This fraction, the homogenate, was centrifuged at 300,000 × *g* for 45 min yielding a supernatant containing soluble phosphatases

Abbreviations: GRP, G-protein-coupled receptor phosphatase; βAR, β_2-adrenergic receptor; α_2C_2AR, α_2C_2-adrenergic receptor; GRK, G-protein-coupled receptor kinase; βARK, βAR kinase; RK, rhodopsin kinase; PKA, cAMP-dependent protein kinase; PP-2A, protein phosphatase type 2A; PP-1, protein phosphatase type 1; OA, okadaic acid; munit(s), unit(s) × 10^{-3}.

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(the soluble fraction) and a pellet of membrane-enriched particulate matter. The particulate material was rehomogenized in buffer B containing 500 mM NaCl and extracted on ice for 30 min. Centrifugation at 300,000 × *g* for 45 min yielded a soluble salt-wash fraction that was concentrated and desalted. The remaining particulate material was resuspended in 25 ml of buffer B and is termed the particulate fraction.

Gel Filtration of GRP Activity. The particulate fraction from bovine brain was incubated with 0.25% dodecyl β-maltoside in buffer B at 4°C for 45 min. Soluble proteins were recovered after centrifugation at 350,000 × *g* for 10 min. Where indicated, detergent was removed from this preparation by using Extracti-Gel (Pierce) (5). Gel filtration was performed on a Superose-12 column (10 × 300 mm) in 20 mM Tris·HCl, pH 7.0/2 mM EDTA/5% (vol/vol) glycerol/100 mM NaCl in the presence or absence of 0.05% dodecyl β-maltoside. The column was developed at a flow rate of 0.3 ml/min and 0.3-ml fractions were collected.

Immunoblot Analysis. Western blot analysis of GRP-containing fractions was performed by using rabbit antisera directed against the catalytic subunit (14), the structural A subunit (15) and regulatory B subunits [B_α (16), B_β (16), and B′ (17)] of PP-2A, and the ECL kit (Amersham). Purified PP-2A (16) and a lysate of Sf9 cells overexpressing the B_β subunit were used as positive controls.

RESULTS AND DISCUSSION

The Principle GRP Activity of Bovine Brain Is a Latent Form of PP-2A. A homogenate of bovine brain was prepared and assayed for its ability to dephosphorylate βARK-phosphorylated βAR. Surprisingly, negligible spontaneous receptor phosphatase activity was observed (Fig. 1), although both PP-1 [0.28×10^{-3} unit (munit)/mg of protein] and PP-2A (0.5 munit/mg of protein) activities could be detected by using phosphorylase *a* as a substrate. Similarly, negligible receptor phosphatase activity was detected when assays were performed in the presence of 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, or Ca^{2+}/calmodulin (0.1 mM/0.2 μM), indicating that the cation-dependent phosphatases PP-2C and PP-2B do not play a role in dephosphorylating this substrate (Fig. 1*B*). Potent GRP activity was, however, observed upon addition of protamine or after a freeze/thaw treatment of the extract in the presence of 2-mercaptoethanol (Fig. 1). These treatments have been shown to potently activate PP-2A (18). The initial rate of dephosphorylation of the βAR when assayed in the presence of protamine after a freeze/thaw treatment was ≈0.4 mol of P_i released per mol of βAR per min with 75% of the substrate being dephosphorylated after 10 min. Addition of I-2 (100 nM), a potent and selective inhibitor of PP-1, inhibited 40% of the total phosphorylase *a* phosphatase activity but had no effect on either the protamine or freeze/thaw-stimulated receptor phosphatase activity (Fig. 1). Furthermore, OA potently inhibited freeze/thaw-activated GRP activity with an IC_{50} of 0.1 nM (data not shown), consistent with reported values for the catalytic subunit of PP-2A (19). The GRP activity of bovine brain would, thus, appear to be a low-activity oligomeric form of PP-2A.

The GRP Activity Is Membrane-Associated. To assess the intracellular distribution of the receptor phosphatase, soluble, particulate, and salt-wash fractions were prepared from the bovine brain homogenate and assayed for GRP activity. The receptor phosphatase activities of the fractions were assayed at approximately equivalent phosphorylase *a* phosphatase activities (0.05 munit/ml). As described (Fig. 1) negligible receptor phosphatase activity was observed in any fraction in the absence of activators of PP-2A. Freeze/thaw treatment in the presence of 2-mercaptoethanol revealed significant GRP activity in the homogenate, which was exclusively associated with the particulate fraction (Fig. 2). The latency and membrane localization of the GRP distinguish this enzyme from the spontaneously active soluble PP-2A reported to dephosphorylate rhodopsin (20–22) and the cholecystokinin receptors (23). However, previous studies failed to assess the receptor phosphatase activity of the salt-washed particulate fraction. Additionally, when assessing cholecystokinin receptor dephos-

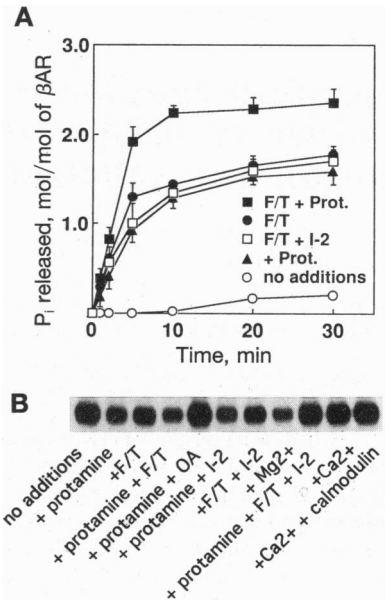

FIG. 1. GRP activity of a bovine brain homogenate. (*A*) GRP assays were performed with no additions (○) or in the presence of protamine sulfate (1 mg/ml) (▲). After freeze/thaw treatment (F/T) of the homogenate, assays were performed in the presence (■) or absence (●) of protamine sulfate (Prot., 1 mg/ml) or in the presence of 100 nM I-2 (□). Assays were performed at a phosphorylase *a* phosphatase activity of 0.1 munit/ml. The results shown represent the mean ± SEM for three experiments. (*B*) Autoradiograph showing the effects of various treatments on GRP activity. Samples were incubated for 30 min at 30°C and subsequently electrophoresed on SDS/10% polyacrylamide gels. The band corresponding to phosphorylated βAR is shown. Assays contained the following additions, where indicated: protamine, 1 mg/ml; I-2, 100 nM; OA, 5 μM; Mg^{2+}, 5 mM; Ca^{2+}, 0.1 mM; Ca^{2+}/calmodulin, 0.1 mM/0.2 μM. The effect of freeze/thaw treatment of the homogenate is also shown (F/T).

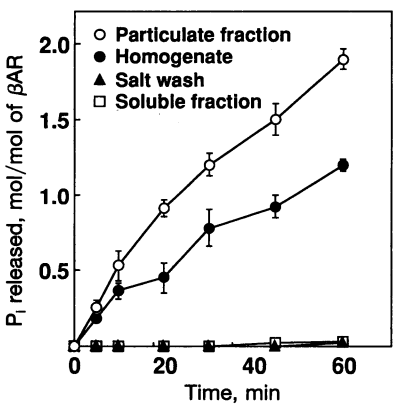

FIG. 2. Receptor phosphatase activity is associated with the particulate fraction. The GRP activities of a bovine brain homogenate (●) and three fractions derived from this extract, a soluble fraction (□), a salt-wash fraction (▲), and a particulate fraction (○), were compared. Fractions were subjected to freeze/thaw treatment in the presence of 0.2 M 2-mercaptoethanol prior to assay. The extract, soluble, and salt-extracted fractions were assayed at a spontaneous phosphorylase *a* phosphatase activity of 0.1 munit/ml, and the particulate fraction was assayed at a phosphorylase *a* phosphatase activity of 0.05 munit/ml. The data shown represent the mean ± SEM of three experiments.

phorylation, pancreatic cells were lysed and phosphatase assays were performed in the presence of detergent, conditions favoring dissociation of the latent GRP (23). A role for the latent membrane-associated GRP in the dephosphorylation of these receptors (rhodopsin and cholecystokinin) cannot, therefore, be discounted.

The particulate localization of the GRP was a somewhat surprising finding since PP-2A is generally considered to be a soluble enzyme. That PP-2A phosphatase activity was indeed present in the particulate fraction was confirmed by using phosphorylase *a* as a substrate. OA and I-2 were used to determine the relative activities of PP-1 and PP-2A in each of the fractions assayed for GRP (Table 1). As expected, the bovine brain homogenate contained approximately equal amounts of spontaneously active PP-1 and PP-2A phosphatase activity. The soluble fraction was predominantly PP-2A (85%) and the salt-extracted fraction PP-1 (85%). The particulate fraction contained little spontaneous phosphatase activity (0.25 munit/mg of protein).

Freeze/thaw treatment under reducing conditions dissociates oligomeric forms of PP-2A and leads to an activation of this enzyme (18). This treatment thus activates the PP-2A in both the homogenate and the soluble fractions (Table 1). Interestingly, the particulate fraction, which contains the GRP activity, but very little spontaneously active PP-2A, displays the largest fold increase in PP-2A-like activity upon freeze/thaw treatment. After freeze/thaw activation, the phosphorylase *a* phosphatase activity of this fraction increases ≈9-fold. Thus a pool of latent PP-2A, including the GRP, is tightly associated with the particulate fraction.

The presence of latent PP-2A and PP-1 in a crude synaptic plasma membrane preparation derived from rat forebrain was recently reported (24). Extraction of the membranes with 500 mM NaCl and 0.3% Triton X-100 resulted in the elution and activation of both enzymes (24). In this study, treatment of the membrane fraction with 500 mM NaCl alone released membrane-associated PP-1 but not PP-2A activity (Table 1), suggesting different mechanisms of membrane association for these two classes of serine/threonine protein phosphatases.

Substrate Specificity of GRP. To investigate the receptor specificity of the GRP activity, the ability of this enzyme to dephosphorylate three GRK-phosphorylated G-protein-coupled receptors was investigated. Purified Sf9 cell membranes containing βARK-phosphorylated βAR or α_2C_2AR or rod outer segment membranes containing RK-phosphorylated rhodopsin were utilized as substrates for the GRP. All three GRK-phosphorylated receptors were dephosphorylated in an I-2-resistant fashion after freeze/thaw activation of the particulate fraction (Fig. 3*A*). The GRP would thus appear to have a broad specificity for GRK-phosphorylated G-protein-coupled receptors.

Does the GRP exhibit specificity for particular phosphorylation sites? To examine this question, the ability of the salt-washed particulate fraction to dephosphorylate GRK- or PKA-phosphorylated βAR substrates was examined. βARK-1,2 and RK but not PKA-phosphorylated βAR served as substrates for the activated GRP (Fig. 3*B*). Dephosphorylation was unaffected by I-2 addition. Thus the membrane-associated GRP dephosphorylates a number of G-protein-coupled receptors; moreover, for the βAR, those sites phosphorylated by GRKs rather than PKA appear to be the preferred substrates for this enzyme.

Table 1. Distribution of PP-1 and PP-2A enzyme activities in bovine brain extracts

	Phosphorylase *a* phosphatase activity, munits/mg of protein			
	Freshly prepared		Freeze/thaw activated	
Extract	PP-2A	PP-1	PP-2A	PP-1
Homogenate	0.5	0.281	2.75	0.35
Soluble	1.13	0.198	2.1	0.3
Salt wash	0.495	2.81	0.46	1.89
Particulate	0.125	0.125	2.08	0.17

Samples were assayed before or after freeze/thaw treatment. The results shown represent the mean of six determinations performed on three bovine brain preparations.

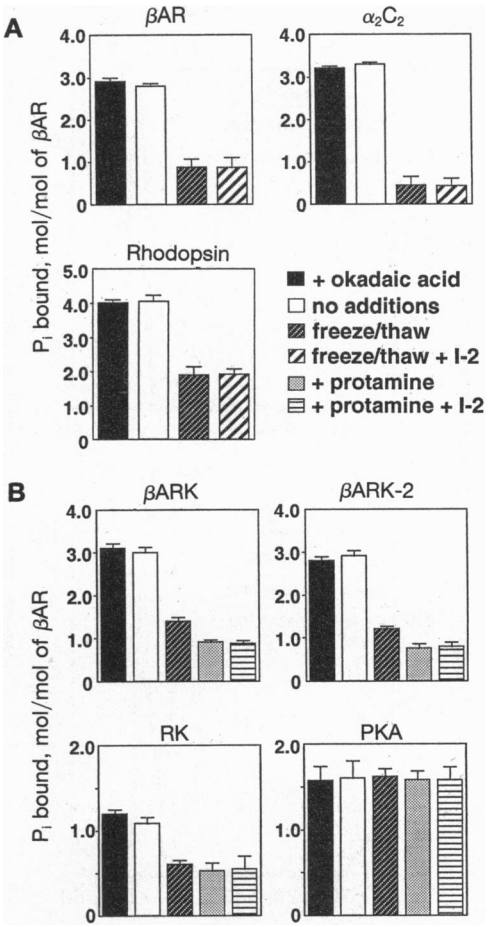

FIG. 3. Substrate specificity of the GRP. (*A*) The GRP activity of the particulate fraction assayed against βARK-phosphorylated βAR, βARK-phosphorylated α_2C_2AR, and RK-phosphorylated rhodopsin. Assays were performed by using untreated particulate material in the presence or absence of 5 μM OA (+ okadaic acid, no additions) or using freeze/thaw-treated particulate material in the presence or absence of 100 nM I-2. The receptor substrates are indicated. All assays were performed at a phosphorylase *a* phosphatase activity of 0.05 munit/ml. The data shown represent the mean ± SEM for three experiments. (*B*) GRP activity of a salt-washed particulate fraction assessed by using as substrates purified reconstituted βAR phosphorylated with βARK (2.7 ± 0.3 mol of P_i per mol of βAR), βARK-2 (3.1 ± 0.5 mol of P_i per mol of βAR), RK (1.5 ± 0.5 mol of P_i per mol of βAR), or PKA (1.6 ± 0.1 mol of P_i per mol of βAR). Assays were performed at a phosphorylase *a* phosphatase activity of 0.05 munit/ml by using untreated particulate material in the presence or absence of 5 μM OA or after freeze/thaw treatment of the phosphatase preparation, in the presence of protamine (1 mg/ml), or in the presence of protamine (1 mg/ml) and 100 nM I-2. The data shown represent the mean ± SEM for three experiments.

Resolution of Two Forms of the GRP by Gel Filtration. To gain information concerning the structure of the GRP, detergent-solubilized preparations derived from the particulate fraction of bovine brain were subjected to gel filtration on Superose-12 (Fig. 4). The solubilized preparation exhibited spontaneous GRP activity when assayed in the presence of detergent (Fig. 4*A*). Removal of detergent, however, resulted

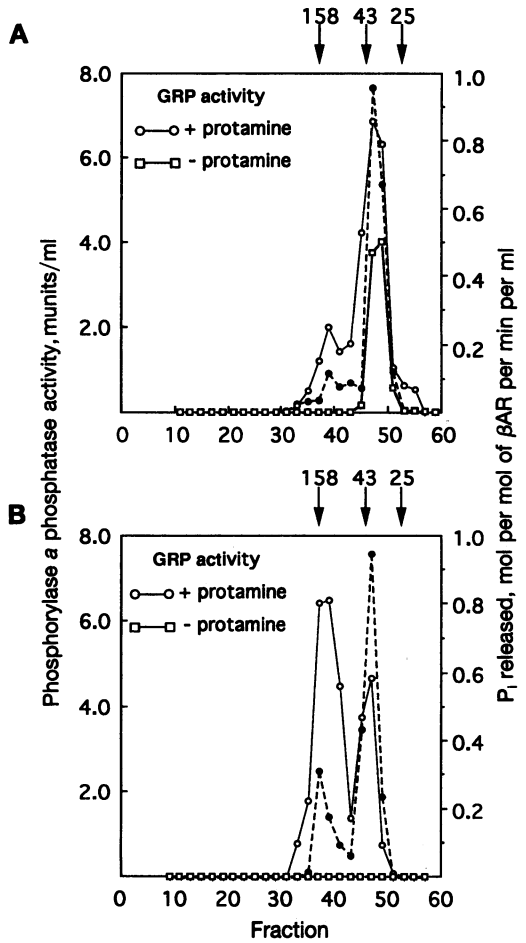

FIG. 4. Gel filtration of the receptor phosphatase activity. The detergent-solubilized particulate fraction was subjected to gel filtration on a Superose-12 column in the presence of detergent (0.05% dodecyl β-maltoside) (*A*). Alternatively, prior to gel filtration, detergent was removed from the solubilized fraction and gel filtration was performed in the absence of detergent (*B*). Fractions were assayed for phosphorylase *a* phosphatase (●) and GRP activity. GRP activity was measured in the presence (○) or absence (□) of protamine sulfate (1 mg/ml). The elution positions of aldolase (158 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) are indicated.

in the formation of a GRP activity with properties similar to those of the native enzyme; i.e., the enzyme was inactive in the absence of protamine (Fig. 4*B*). Gel filtration, in the presence of detergent, revealed a major peak of both phosphorylase *a* phosphatase and GRP activities. The receptor phosphatase activity was spontaneous, inhibited by 2 nM OA, and stimulated by protamine. Furthermore, by gel filtration, it had an apparent molecular mass of 35 kDa, observations consistent with the identification of the catalytic subunit of the GRP as the catalytic subunit of PP-2A (Fig. 4*A*).

Gel filtration of the solubilized latent form of the GRP (i.e., in the absence of detergent) revealed no spontaneous activity (Fig. 4*B*). However, two peaks of GRP activity, with apparent molecular masses of 150 kDa and 35 kDa, were observed when the fractions were assayed in the presence of protamine (Fig. 4*B*). Thus removing detergent appears to promote the reassociation of the catalytic subunit of the GRP with one or more regulatory subunits. At equivalent phosphorylase *a* phosphatase activities, the receptor phosphatase activity of the oligomeric enzyme is ≈4-fold higher than that of the free catalytic subunit.

The free catalytic subunit of the GRP displays spontaneous receptor phosphatase activity when assayed in the presence but not the absence of detergent (Fig. 4). This apparent difference in GRP latency is specific to the βAR substrate since the phosphorylase *a* phosphatase activity of the 35-kDa GRP is similar in either the presence or absence of detergent. Thus receptor conformation appears to play a role in regulating receptor dephosphorylation. The latency of the GRP may thus potentially be overcome by direct activation of this enzyme or, alternatively, by alteration of the conformation of its receptor substrates.

In a previous study (25), the ability of various purified preparations of phosphatase catalytic subunits to dephosphorylate the βAR was investigated. Of the purified phosphatase catalytic subunits utilized, PP-2A, PP-1, PP-2B, and the catalytic subunit of a latent high molecular weight phosphatase LP-2, only the latter was capable of dephosphorylating βARK-phosphorylated βAR. Although LP-2 shares some properties with the GRP—namely, its latency—it is a soluble enzyme with a subunit molecular mass of 49 kDa and, thus, seems unlikely to account for the GRP activity of bovine brain homogenates.

Immunological Analysis of the GRP. PP-2A holoenzymes have been demonstrated to exist as a heterotrimeric complex consisting of a 36-kDa catalytic subunit (C), a 65-kDa structural subunit (A), and a third regulatory subunit ranging in molecular mass between 52 and 74 kDa (26). In an attempt to gain information concerning the relationship between the GRP and previously characterized forms of PP-2A, fractions containing the 150-kDa GRP activity, (Fig. 4*B*, fractions 36–38) were pooled and subjected to immunoblot analysis. Polyclonal antibodies specific for the catalytic (36 kDa), A (65 kDa), and three distinct B subunits [B_α (55 kDa), B_β (55 kDa), and B′ (53 kDa)] of PP-2A were utilized. As shown in Fig. 5, immunoreactive species of appropriate molecular masses were observed in the GRP fraction blotted with either the catalytic subunit (Fig. 5*A*) or A subunit (Fig. 5*B*) antibodies. Of the three B-subunit antibodies utilized, only that raised against a peptide corresponding to residues 14–26 of the human PP-2A

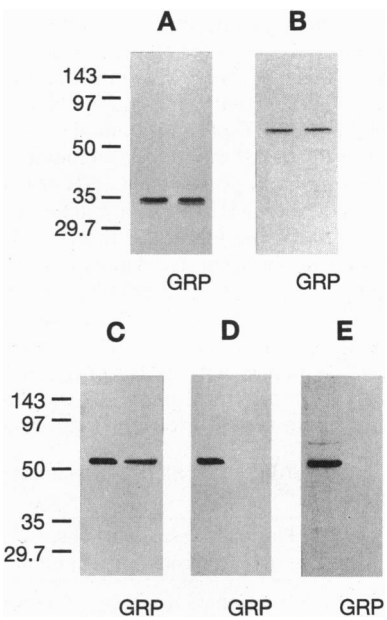

FIG. 5. Immunoblot analysis of the 150-kDa GRP activity. Fractions containing the large molecular mass form of the GRP activity (Fig. 4*B*, fractions 36–38) were pooled and subjected to Western blot analysis with rabbit antisera directed against the catalytic subunit (anti-C) (*A*), the structural A subunit (anti-A) (*B*), and three regulatory B subunits, B_α (anti-B_α) (*C*), B_β (anti-B_β) (*D*), and B′ (anti-B′) (*E*) of PP-2A. Each panel contains two lanes, a control lane (unlabeled) for the antisera containing the protein against which the antibody is directed and a lane containing the pooled GRP fractions (labeled GRP).

subunit B_α displayed immunoreactivity with a protein of appropriate molecular mass in the GRP-containing fraction (Fig. 5*C*). These results support the identification of the GRP as an oligomeric form of PP-2A and suggest a potential subunit structure of $AB_\alpha C$. Interestingly, PP-2A of this subunit composition, the most prevalent soluble form of PP-2A in bovine brain extracts (27), was recently identified as the principle soluble rhodopsin phosphatase of rod outer segments (28). However, we observe negligible soluble GRP activity. Thus although apparently composed of immunologically similar subunits, two prominent characteristics distinguish the GRP from soluble bovine brain PP-2A—namely, its particulate localization and substrate specificity. What potentially could account for these differences? The B_α subunit antibodies utilized in this study were raised against a short peptide sequence derived from this protein (16). The GRP may thus contain a regulatory B subunit highly related to but distinct from B_α. Alternatively, since two isoforms (α and β) of the C and A subunits of PP-2A have been identified (29–31), the GRP and soluble PP-2A may have different isoform compositions. Finally, covalent modifications of the catalytic subunit of PP-2A have been reported including phosphorylation (32, 33) and carboxyl methylation (34). Differential post-translational modification of one or more of the constituent subunits of the GRP may account for the altered properties of this enzyme.

In conclusion, GRP, a latent membrane-associated form of PP-2A representing the principle βAR phosphatase activity of bovine brain, has been identified. Immunological analysis suggests a potential subunit structure for the GRP of $AB_\alpha C$; however, significant differences exist between the GRP and the previously characterized soluble PP-2A of this composition. The reasons for the particulate localization of the GRP remain obscure; however, the latency and localization of this enzyme suggest that it may represent an important regulatory target for modulating the phosphorylation state of membrane-associated receptors.

We thank Dr. Marc C. Mumby (University of Texas, Dallas) for PP-2A antibodies; Darrell Capel for purified βARK-1, βARK-2, and RK; and Grace Irons for virus and cell culture. This work was supported in part by grants from the National Institutes of Health, Grants HL16037 to R.J.L. and DK36569 and P01 HL06308, subprogram 4, to A.A.D.-R.

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